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13. ABSTRACT (Maximum 200 Words) <p>Bone is the most common site of metastases by human breast cancer. Most breast cancers form osteolytic metastases, in contrast to tumors such as prostate cancer that form osteosclerotic metastases. Although some evidence suggests that formation of bone metastases by breast cancer cells is mediated by the increased osteoclastogenesis at the target site, a clear controversy exists whether formation of bone metastases is mediated by breast cancer cells directly or by stimulated osteoclasts. We have therefore examined the expression of RANKL, an important protein involved in the bone remodeling, in invasive carcinoma of breast and bone metastases. We observed that RANKL is present not only in non-neoplastic breast but also in Infiltrating Ductal Carcinoma (IDC). Further, breast cancer cells lose the expression of RANKL as they become metastatic to bone. Therefore the formation of osteolytic lesions in bone by breast cancer cells may not be due to direct interaction of tumor cells and bone. Rather a different mechanism might be operating. However, loss of RANKL expression in Bone metastasis might serve as an indicator of bone metastasis and RANKL might prove as a diagnostic or prognostic marker for breast cancer metastasis.</p>				
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Introduction

Bone is one of the most common target sites for cancer metastasis. Tumors such as breast carcinoma avidly metastasize to bone to form osteolytic lesions. Two models have been postulated to explain the bone destruction associated with breast cancer metastasis: the osteolysis is mediated either directly by tumor cells, or indirectly by osteoclasts. Breast cancer cells induce osteoclast differentiation and proliferation by secreting several osteotropic factors such as IL-1, IL-6, LIF, prostaglandin E2, tumor necrosis factor- α and parathyroid hormone related protein (PTHrP) or by direct cell-cell interaction with bone marrow cells (Mundy 1997, Guise, 1997, Guise and Mundy 1998, Thomas et, 1999). Recently, Receptor Activator of NF- κ B ligand (RANKL) and its receptor RANK, have been identified and implicated in regulation of bone remodeling (Hofbauer et al., 2001). RANKL is expressed on stromal cells and osteoblasts and is thought to mediate the interaction between these cells and pre-osteoclasts by binding to its receptor RANK which is expressed on the pre-osteoclasts. This interaction leads to osteoclastogenesis and bone resorption (Hofbauer et al., 2001). Thus the RANK and RANKL interaction appears to play a key role in the process of osteoclastogenesis and bone resorption. Expression of RANKL in other tumor types such as prostate cancer has earlier been reported (Brown et al., 1999). In addition, studies in mouse model system have also generated evidence for the involvement of RANKL in metastatic bone destruction. (Guise 1997, Guise and Mundy 1998). Therefore my hypothesis was that in breast carcinoma expression of RANKL might correlate with bone metastasis and osteolysis.

Specific Aims:

- To examine different histological forms of breast carcinoma as well as metastatic breast cell lines for expression of RANKL and correlate this expression with phenotype.
- If RANKL expression is detected, to test the ability of anti-RANKL antibodies to inhibit the formation of osteolytic lesions in a model system

Body

It is well established that breast cancers have the capability to establish and grow as metastases in bone, however, the mechanism underlying their ability to induce osteolysis remains uncertain. *In vitro* studies have demonstrated that breast cancer cells alone have the capacity to degrade the bone matrix, although these lesions of bone or dentine slices are not of the magnitude of those resulting from osteoclast-mediated bone destruction (Eilon and Mundy, 1978). Studies have also shown that PTHrP is expressed by the metastatic breast cancer cells and is a critical component in the mechanism of breast cancer metastases to bone (Boyce et al., 1999; Chirgwin and Guise, 2000; de la Mata et al., 1995; Guise, 1997; Guise and Mundy, 1996; Guise et al., 1996; Guise et al., 1993; Henderson et al., 2001; Kohno et al., 1994; Thomas et al., 1999; Uy et al., 1995; Uy et al., 1997; Yoshida et al., 2000). Co-culture experiments have shown that breast cancer cells can produce both PTHrP and M-CSF which induce RANKL mRNA levels and inhibit OPG mRNA levels in osteoblasts *in vitro* (Mancino et al., 2001; Thomas et al., 1999).

Specific Aim 1: Expression of RANKL in Breast Tumors and tumor cell lines:

1a: RANKL expression in Breast tumors:

We have investigated the expression of RANKL on an array of 60 primary and 43 bone metastatic breast tumors by immunohistochemistry using anti-RANKL antibodies. These arrays of infiltrating ductal carcinoma (IDC) were obtained from Imgenex, (San Diego, CA) while bone metastatic tumors were obtained from the Department of Pathology, University of Connecticut Health Center, Fox Chase Cancer Center, Philadelphia and Cooperative Human Tissue Network (CHTN). Samples were prepared from paraffin embedded archival samples. They were cut using microtome and spread on polylysine coated slides. Tumors were stained using the standard protocol (Herrington and McGee, 1992). Briefly slides were deparaffinized with xylene, dehydrated in alcohol and treated with 4N HCl at 37° C for 10 min. to retrieve the antigen. Samples were washed in distilled water and stained with anti-RANKL antibodies using Histostain-SP kit (Zymed, South San Francisco, CA). Samples were mounted and photographed under the microscope. The same batch of tumors was also stained with H&E. In our initial studies, we had reported that both primary Infiltrating Ductal Carcinoma (IDC) as well as one metastatic tumor overexpressed RANKL in comparison to non-neoplastic breast (see annual report May 2002). Upon Examination of the sample size we realized that this was not the case. Both non-neoplastic breast (NNB) as well as IDC express RANKL in epithelial cells whereas expression of RANKL was completely diminished in osteolytic lesions of bone metastasis. (Fig 1-3, Table 1). We then correlated loss of RANKL expression and metastatic phenotype statistically using Chi square and Fisher Exact test (Table 1). In a comparison of NNB with breast cancer bone metastases, loss of RANKL expression strongly correlated with bone metastasis ($p < 0.0001$). In a comparison of metastatic with non-metastatic IDC, loss of RANKL expression correlated with metastasis ($p = 0.012$). Comparison of NNB with IDC revealed that loss of RANKL expression was nearly significant with non-metastatic IDC ($p = 0.0510$ and highly significant with metastatic IDC ($p < 0.0001$). Overall, regardless of stage, loss of expression of RANKL correlated with metastasis ($p < 0.0001$)

11b: Expression of RANKL in Breast Cancer Cell lines:

We next examined the expression of RANKL in MCF-7, T47-D and HCC-38 (Non metastatic) and MDA-MB-231, MDA-MB-435S and ZR.75-1 (metastatic) breast cancer cell lines. All the cell lines had low to no expression of RANKL.

In our preliminary study (annual report 2002) we had observed that normal breast epithelial cells as well as primary and one metastatic breast tumor expressed RANK and RANKL on their cell surface. Based upon that we proposed that for the bone metastasis to take place, breast tumor cells could upregulate RANKL which could interact with osteoclasts to form an osteolytic lesion (Figure 4 Model 1) or breast tumor cells could interact with osteoblasts directly due to the presence of RANK on the tumor cells and RANKL on the osteoblasts to initiate osteolysis (Figure 4 Model 2). However, based upon the larger sample size in this investigation we observe that RANKL is lost in metastatic IDCs, bone metastatic tumors and breast cancer cell lines. Thus, the formation of osteolytic lesions in the bone metastasis of breast may not be by the direct interaction between tumor cells and bone. Rather, loss of expression of RANKL in metastatic tumors might act as a diagnostic marker for the bone metastasis.

Specific Aim II:

In order to accomplish specific aim two we needed to perform in vitro bone resorption assays which required coculturing of tumor cells with dentine bone slices. Since we observed very weak expression of RANKL in breast cancer cell lines these experiments were not possible to perform. In order to accomplish this aim we would need to generate breast cancer cell lines from non-neoplastic breast as well as IDC with high RANKL expression. That will constitute a separate grant application.

KEY RESEARCH ACCOMPLISHMENTS:

- I demonstrated that Receptor Activator of NF- κ B ligand (RANKL) is expressed in benign breast as well as in IDC
- I found that RANKL expression was lost in bone metastasis of breast cancer
- I discovered that expression of RANKL is inversely related to the metastatic phenotype of breast cancer
- Loss of RANKL expression might act as diagnostic indicator of breast cancer to establish in bone

REPORTABLE OUTCOMES:

The data reported here is currently under preparation and will be submitted for publication.

CONCLUSION:

Breast cancer cells have the capability to establish and grow as metastasis in bone, however, the mechanism underlying their osteolysis is not understood. A controversy exists whether tumor cells are capable of osteolysis by themselves or is this mechanism mediated by osteoclasts. In the present investigation we have observed that RANKL is present not only in benign breast but also in IDC. Further, breast cancer cells lose the expression of RANKL as they become metastatic to bone. Therefore the formation of osteolytic lesions in bone by breast cancer cells may not be due to direct interaction of tumor cells and bone. Rather a different mechanism might be operating. However, onset of loss of RANKL expression might be an indicator of onset of bone metastasis.

REFERENCES

- Boyce, B. F., Yoneda, T., and Guise, T. A, *Endocr Relat Cancer* **6**, 333 (1999)
- Brown J. M., Quinn J. E., Buhler K. R., Vessella R. L, *J Bone Miner Res* **14**(Suppl 1), 1085 (2000)
- Chirgwin, J. M., and Guise, T. A, *Crit Rev Eukaryot Gene Expr* **10**, 159 (2000)
- Coleman, R. E., and Rubens, R. D, *Br J Cancer* **55**, 61 (1987)
- De la Mata, J., Uy, H. L., Guise, T. A., Story, B., Boyce, B. F., Mundy, G. R., and Roodman, G. D, *J Clin Invest* **95**, 2846 (1995)
- Eilon, G., and Mundy, G. R, *Nature* **276**, 726 (1978)
- Guise, T. A, *Cancer* **80**, 1572 (1997)
- Guise, T. A., and Mundy, G. R, *Curr Opin Nephrol Hypertens* **5**, 307 (1996)
- Guise, T. A., Yin, J. J., Taylor, S. D., Kumagai, Y., Dallas, M., Boyce, B. F., Yoneda, T., and Mundy, G. R, *J Clin Invest* **98**, 1544 (1996)
- Guise, T. A., Yoneda, T., Yates, A. J., and Mundy, G. R., *J Clin Endocrinol Metab* **77**, 40 (1993)
- Henderson, M., Danks, J., Moseley, J., Slavin, J., Harris, T., McKinlay, M., Hopper, J., and Martin, T, *J Natl Cancer Inst* **93**, 234 (2001)
- Herrington C.S. and McGee, J.O' D., *Diagnostic Molecular Pathology* (IRL, New York pp 8-17, 1992)
- Holfbauer, L C., Neubauer, A ., and Heufelder, A.E, *Cancer* **92**,460 (2001)
- Hunt, N. C., Fujikawa, Y., Sabokbar, A., Itonaga, I., Harris, A., and Athanasou, N. A, *Br J Cancer* **85**, 78 (2001)
- Kohn, N., Kitazawa, S., Sakoda, Y., Kanbara, Y., Furuya, Y., Ohashi, O., and Kitazawa, R, *Breast Cancer* **1**, 43 (1994).
- Mancino, A. T., Klimberg, V. S., Yamamoto, M., Manolagas, S. C., and Abe, E., *J Surg Res* **100**, 18 (2001)
- Thomas, R. J., Guise, T. A., Yin, J. J., Elliott, J., Horwood, N. J., Martin, T. J., and Gillespie, M. T, *Endocrinology* **140**, 4451 (1999)
- Uy, H. L., Guise, T. A., De La Mata, J., Taylor, S. D., Story, B. M., Dallas, M. R., Boyce, B. F., Mundy, G. R., and Roodman, G. D, *Endocrinology* **136**, 3207 (1995)
- Uy, H. L., Mundy, G. R., Boyce, B. F., Story, B. M., Dunstan, C. R., Yin, J. J., Roodman, G. D., and Guise, T. A, *Cancer Res* **57**, 3194 (1997)
- Yin, J. J., Selander, K., Chirgwin, J. M., Dallas, M., Grubbs, B. G., Wieser, R., Massague, J., Mundy, G. R., and Guise, T. A, *J Clin Invest* **103**, 197 (1999)
- Yoshida, A., Nakamura, Y., Shimizu, A., Harada, M., Kameda, Y., Nagano, A., Inaba, M., and Asaga, T, *Breast Cancer* **7**, 215 (2000)

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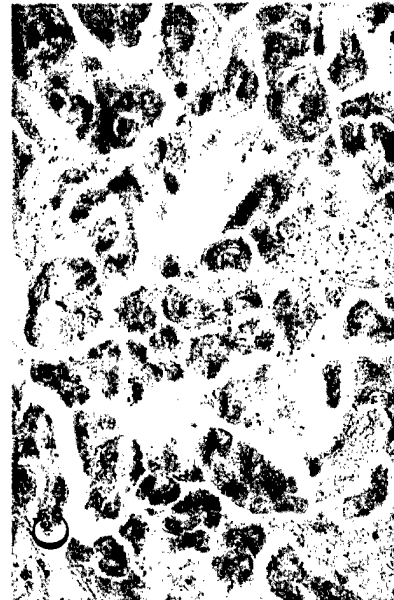
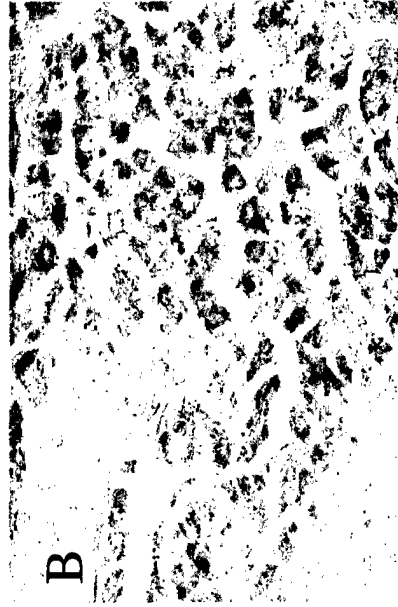
Fig 1: RANKL Expression in Non-Neoplastic Breast

A: Non-Neoplastic Breast H&E 20X

B: Non-Neoplastic Breast RANKL 40X

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**Fig : Infiltrating Ductal carcinoma
Stained with Anti-RANKL antibody**

A H&E

B RANKL (20X)

C RANKL (40X)

Note Tumor cells Positive for RANKL

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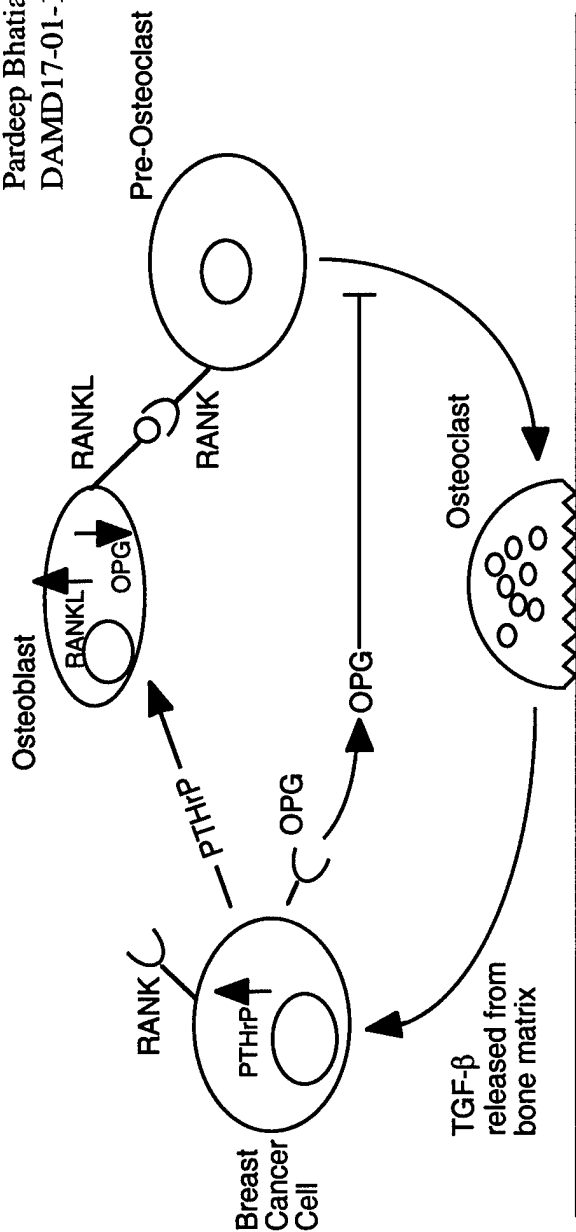


Fig 3: RANKL Expression of Bone metastasis stained with Anti-RANKL anti-body

A: H&E

B: RANKL

C: Negative Control



Model 2:
Breast cancer cells secrete PTHrP that stimulate osteoblasts that then stimulate the breast cancer cells to initiate osteolysis directly

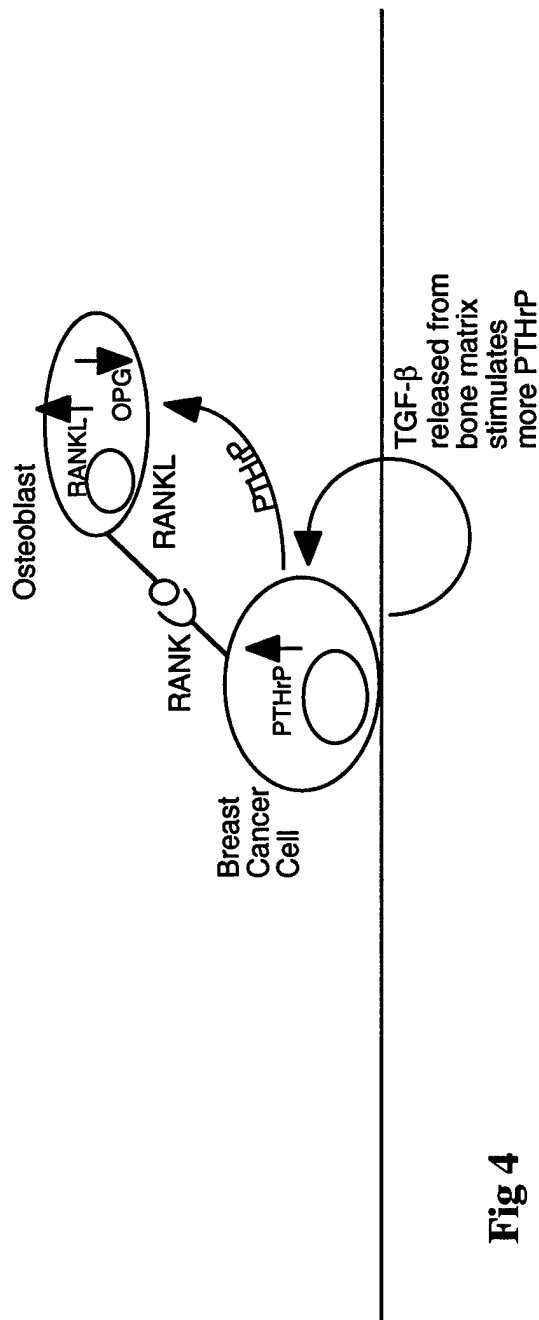


Fig 4

Table: 1

Histology	Disease Status	RANKL Status
Non Neoplastic Breast	-	9/10 Positive
IDC	Non Metastatic	16/26 Positive
IDC	Metastatic	10/32 Positive
Bone Metastasis of Breast	Bone Metastasis	1/43 Positive